# An Evaluation of Biotransformation Products from 2,4-Dinitrotoluene Under Nitrate-Reducing Conditions


## FINAL REPORT

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Wastewater generated during the manufacture of munitions often contains significant levels of nitrates and 2,4-dinitrotoluene (DNT). The objective of this project was to characterize the major biotransformation products formed from DNT under denitrifying conditions, and to identify the organisms responsible. In a denitrifying enrichment culture that used ethanol as the primary substrate, DNT was transformed primarily to 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, and 2,4-diaminotoluene. With extended incubation (≥ 80 days), all of the 2,4-diaminotoluene subsequently disappeared. In cultures that received [$^{14}$C]DNT, nearly all of the labeled metabolites remained in the aqueous phase. Approximately 35% consisted of insoluble material, while 29% was soluble hydrophobic and 32% was soluble hydrophilic. Two organisms were isolated from the enrichment: *Pseudomonas aeruginosa* and a much slower-growing rod. Pure cultures of *P. aeruginosa* only partially reduced DNT to 2,4-diaminotoluene under both aerobic and denitrifying conditions. Accumulation of 2-amino-4-nitrotoluene accounted for 25-45% of the DNT consumed, while 4-acetylamino-2-nitrotoluene accounted for 32-35%. Reduction and acetylation therefore appear to be major biotransformation pathways for DNT under both aerobic and denitrifying conditions.

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## 1.0 STATEMENT OF THE PROBLEM STUDIED

Of the various hazardous waste problems facing the U. S. Army, treatment of wastewater containing 2,4-dinitrotoluene (DNT) is a high priority. Because of its toxicity, DNT is listed by EPA as a priority pollutant. A wastewater discharge limit will soon be established at 113 μg/L. This is an ambitious level, given that some DNT waste streams contain levels as high as 30 to 400 mg/L. DNT is also on EPA's list of compounds that will soon be regulated under the Safe Drinking Water Act Amendments, reflecting the fact that water supplies are increasingly threatened with DNT contamination [9]. Treatment by physical and chemical methods, such as adsorption on activated carbon, have been successfully demonstrated, but the cost is often prohibitively high. Use of biological treatment is an attractive alternative, as long as the products formed are nonhazardous.

Biotransformation of DNT occurs both by oxidation and reduction. The ultimate products of aerobic bio-oxidation are $CO_2$, $NO_2^-$, and cells. With reduction, the products include 2-nitro-4-aminotoluene, 2-amino-4-nitrotoluene, and 2,4-diaminotoluene (DAT). Oxidative and reductive transformations may or may not operate simultaneously. Of the two, oxidation is certainly the preferred route, since this can lead to the most complete destruction of DNT. The biotransformability of the reduced compounds is largely unknown. At least one report has indicated that the reduced nitroaromatics are actually more hazardous than the parent compounds [3].

Oxidation of DNT to $CO_2$ under aerobic conditions has been reported with only two organisms, a pseudomonad strain [10] and *Phanerochaete chrysosporium* [11]. With the pseudomonad, DNT transformation was examined when it was provided as a sole substrate, with stoichiometric release of nitrite. DNT oxidation involved the introduction of molecular oxygen by an oxygenase enzyme. While such studies provide important insights, actual DNT waste streams include other organic compounds. Chief among these are ethanol and ether, which are typically present at concentrations ranging from 100 to 800 mg/L and trace levels to 9,000 mg/L,

respectively. The effect of these readily degradable substrates on aerobic bacterial biotransformation of DNT is not fully understood.

Previous studies on DNT biotransformation under anaerobic conditions indicate that the compound is readily reduced to nitrosonitrotoluenes, aminonitrotoluenes, and 2,4-diaminotoluene (DAT). Stoichiometric reduction of DNT to DAT has been reported under methanogenic conditions, with ethanol serving as the electron donor [1, 5, 6, 12]. No further biodegradation of the DAT was detected, although subsequent aerobic oxidation of DAT was demonstrated in activated sludge [12]. DNT reduction was also reported by Boopathy and Kulpa [2] with a sulfate-reducing *Desulfovibrio* sp., although they recovered only 70% to 82% of the DNT as DAT. Under nitrate-reducing conditions, Liu et al. [4] reported reduction of DNT to nitrosotoluenes and aminonitrotoluenes, but no activity with the same mixed culture under aerobic conditions. No other studies were found where DNT biotransformation was examined during active denitrification.

In addition to nitrosonitrotoluenes and aminonitrotoluenes, DNT biotransformation to a large number of other intermediate metabolites and end products of side reactions is known to occur, although a complete mass balance has not been reported [4, 7]. Knowing the identity and amount of metabolites formed is critical to assessing their toxicity relative to DNT. Our previous work on DNT biotransformation under denitrifying conditions indicated that DNT is initially transformed to aminonitrotoluenes and DAT, and that DAT is subsequently transformed to hydrophilic metabolites, without any significant mineralization [8]. Due to the high concentrations of nitrate usually present in DNT-contaminated waste streams from ammunition plants, it is likely that the transformation of DNT is partially associated with the activity of denitrifying organisms. In order to ascertain the fate of DNT in engineered, as well as natural anoxic environments, it is essential that the main metabolites be fully characterized.

The objective of this project was to characterize the biotransformation products formed from DNT under denitrifying conditions and to identify the denitrifying community responsible for the transformations. A complete mass balance of transformation products was obtained using

[$^{14}$C]DNT. The most important results from the project are described below; a description of the methods used is provided in Appendix 1.

## 2.0 SUMMARY OF THE MOST IMPORTANT RESULTS

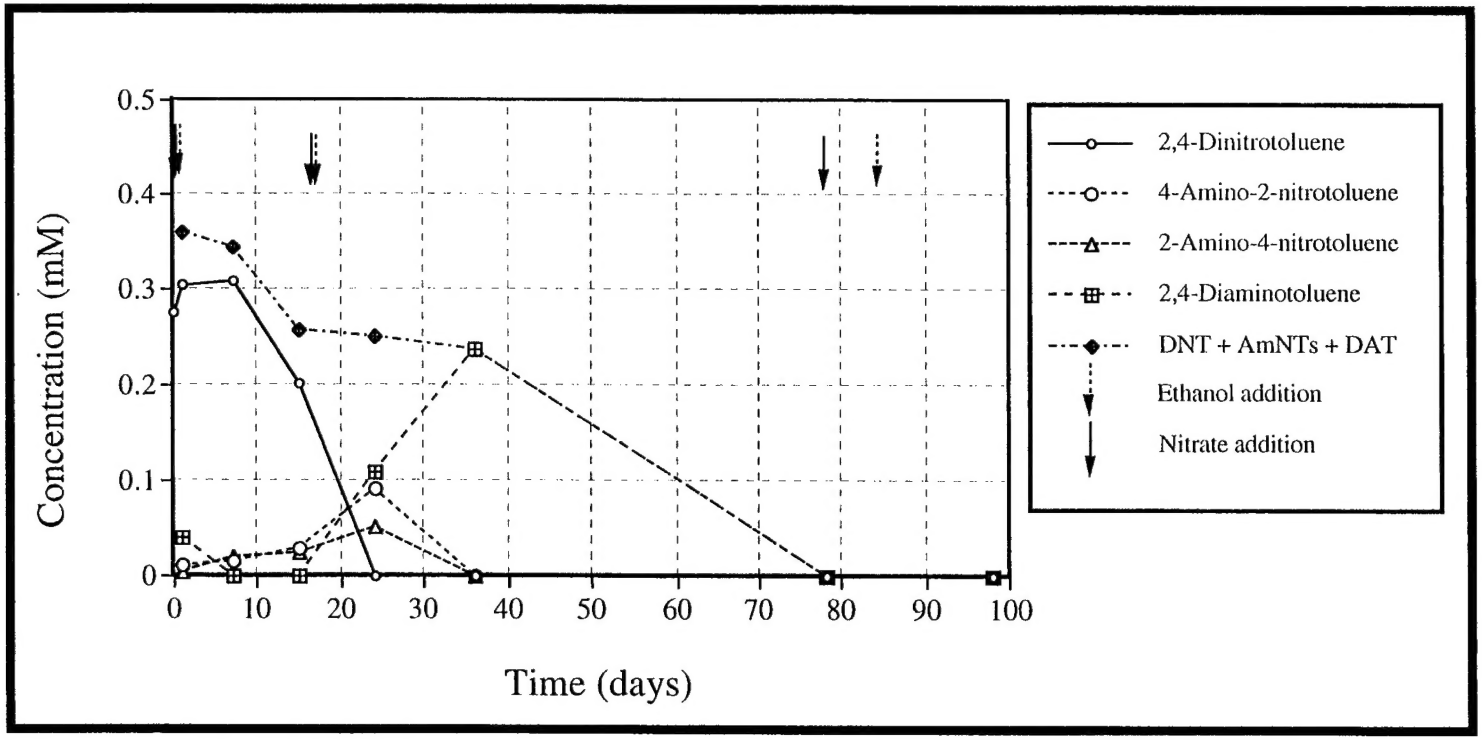
### 2.1 DNT Biotransformation by Denitrifying Enrichment Cultures

Transformation of DNT was observed under denitrifying conditions (Figure 1). Losses of DNT from autoclaved controls were negligible, indicating biological activity was responsible for this transformation. An external electron donor was required to sustain the transformation. Methanol or ethanol was used in this study.

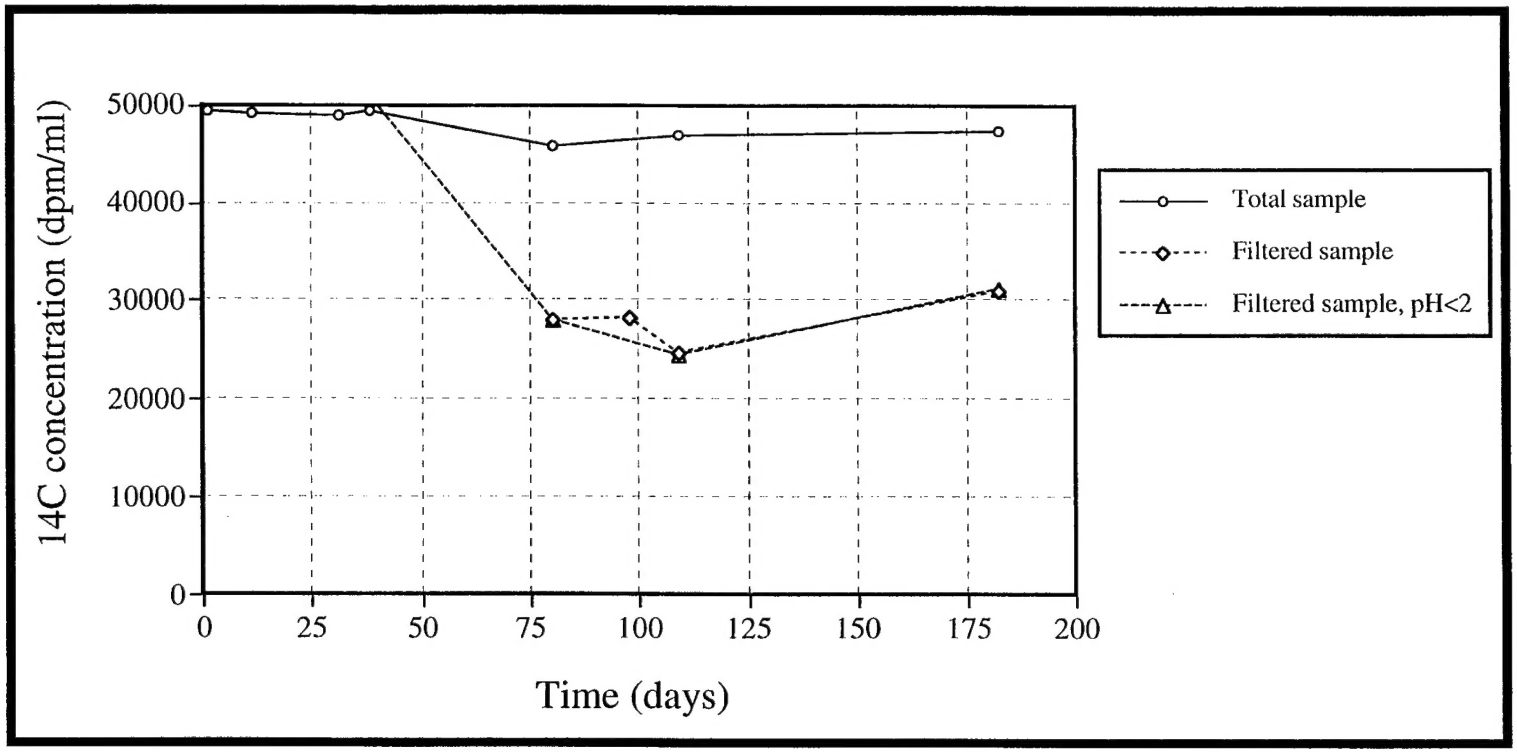
The first biotransformation step was reduction of DNT to 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, and DAT. DAT was further transformed to other products. Figure 1 presents a typical result for DNT transformation by a denitrifying culture enriched from a municipal wastewater treatment plant (Urbana, Illinois). The decline in DNT was accompanied by an accumulation of 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene. Then, DAT was produced from the reduction of the nitro group in the aminonitrotoluenes. After DAT reached a maximum concentration, it too disappeared. The accumulation of other metabolites could not be traced by gas chromatography (GC), since the disappearance of DAT did not result in the appearance of any other peaks in the GC trace. Analysis of samples by high performance liquid chromatography (HPLC) confirmed the disappearance of DAT, which coincided with an increase in the size of the solvent peak. This indicated that the metabolites produced are hydrophilic and are not retained by the hydrophobic stationary phase of the HPLC column. Thus, traditional HPLC or GC techniques could not be relied on to achieve separation of the metabolites.

A uniformly labeled [$^{14}$C]DNT tracer was used to acquire more information on the characteristics of the unidentified metabolites in order to design and optimize other chromatographic techniques necessary for their separation and subsequent identification. Uniformly labeled [$^{14}$C]DNT was added to cultures (50,000 dpm/ml) along with 0.35 mM unlabeled DNT, 80 mM ethanol, and 150 mM nitrate. Figure 2 shows the fate of the $^{14}$C during

# Figure 1. Biotransformation of DNT under nitrogen reducing conditions



# Figure 2. Fate of the radioactive tracer during the biotransformation of DNT



180-days of incubation. Only a small fraction of the $^{14}C$ was found in the head space of the culture bottles (150 dpm/ml of gas). Accounting for the volume of gas produced during the experiment, the amount of $^{14}C$ that volatilized represented only 1.3% of the [$^{14}C$]DNT added. This low amount could be the result of mineralization, production of volatile organic metabolites, or impurities in the [$^{14}C$]DNT stock solution.

Measurements of the aqueous phase indicated that 96% of the tracer remained in the liquid. Filtration of the samples through a 0.45 μm pore size filter retained 35% of the $^{14}C$. The insoluble material retained may be biomass, materials adsorbed into the biomass or filters, or precipitation of metabolites. This fraction has not yet been further characterized. However, since the samples were mixed with methanol prior to filtration, there is a low probability that the $^{14}C$ activity retained on the filter is a consequence of adsorption. In some cases, the cultures developed a change of color that is characteristic of azo compounds, supporting the idea that the insoluble fraction consists of precipitated metabolites. Since there are two potential sites for the formation of azo bonds, it is possible to have condensation of several aromatic groups to form high molecular weight polymers that would have very low solubility. The formation of azo bonds takes place between an amino group and a nitroso group, which is a short lived intermediate in the reduction of the nitro group. The presence of oxygen has been observed to facilitate the formation of the azo bondage. It is possible that some oxygen entered the culture bottles during the extended incubation periods, especially at times when the electron donor (methanol or ethanol) was exhausted.

About 65% of the tracer was soluble (i.e., passed through the 0.45 μm filter). This fraction was further characterized as follows: Only 18% of the radioactive tracer was extractable in methylene chloride and 12% in ether. These results explain, in part, the inadequacy of detecting the DAT metabolites by GC, which requires that samples be concentrated by extraction. The samples were also analyzed for the presence of dissolved $^{14}CO_2$. The mixtures were acidified to pH<2 and bubbled for 5 minutes with $N_{2(g)}$, then analyzed for $^{14}C$ activity. All

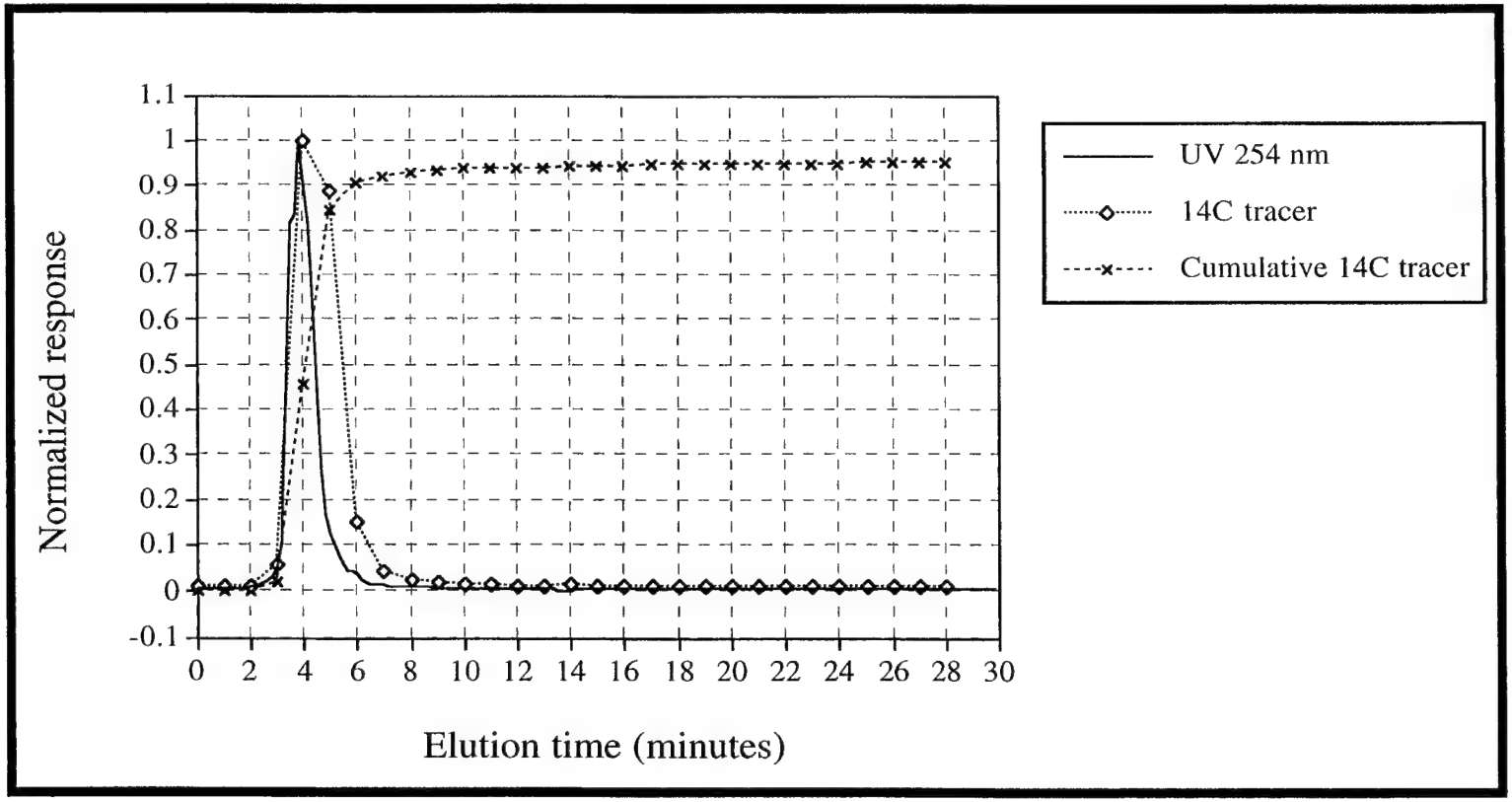
of the tracer remained in the sample, indicating that $^{14}CO_2$ (and other compounds that are purgeable at low pH) was not present.

The relative hydrophobicity of the DNT metabolites was analyzed by HPLC. Fractions of the eluent were collected every minute and analyzed by scintillation for the presence of the radioactive tracer. During an isocratic run with a 50/50 acetonitrile/water eluent, 95% of the tracer was recovered in the solvent front, indicating the hydrophilicity of the metabolites (Figure 3). A gradient elution with the aqueous buffer/methanol system (see Analytical Methods, Appendix 1) was able to separate the radioactive metabolites into two groups (Figure 4): about 45% of the tracer in the soluble fraction (29% of the original tracer) was found in the solvent front (hydrophilic), while the remaining tracer (32% of the original tracer) eluted only when the concentration of methanol was higher than 50% (hydrophobic). For this late eluting fraction, however, no distinct peaks were observed in the UV or $^{14}C$ traces, which suggests the presence of a mixture of several hydrophobic compounds, with none of them at a high enough concentration to produce a distinct peak.

The hydrophilic fraction of the soluble metabolites was further analyzed using a gradient elution that included the addition of 20 ml cetyltrimethylammonium bromide, 1 min prior to injection of the sample. This separated the hydrophilic fraction into three groups (Figure 5). Only 25% of the tracer remained unretained (16% of the original tracer), while the other 20% was retarded by the presence of the ion pairing reagent. Two distinct peaks were observed, which indicated the presence of two different negatively charged compounds. Isolation and identification of these metabolites is still in progress. The unretained part of the hydrophilic metabolites is either neutral or basic, since its retention characteristics were not modified by the positively charged ion pairing reagent.

Figure 6 summarizes the characterization of the DNT metabolites with the radioactive tracer. The quantities given represent the percentage of the original [$^{14}C$]DNT added.

**Figure 3. HPLC analysis of the soluble DNT metabolites. Isocratic run with 50/50 acetonitrile/water eluent**



# Figure 4. HPLC analysis of the soluble DNT metabolites. Gradient elution with aqueous buffer/methanol system

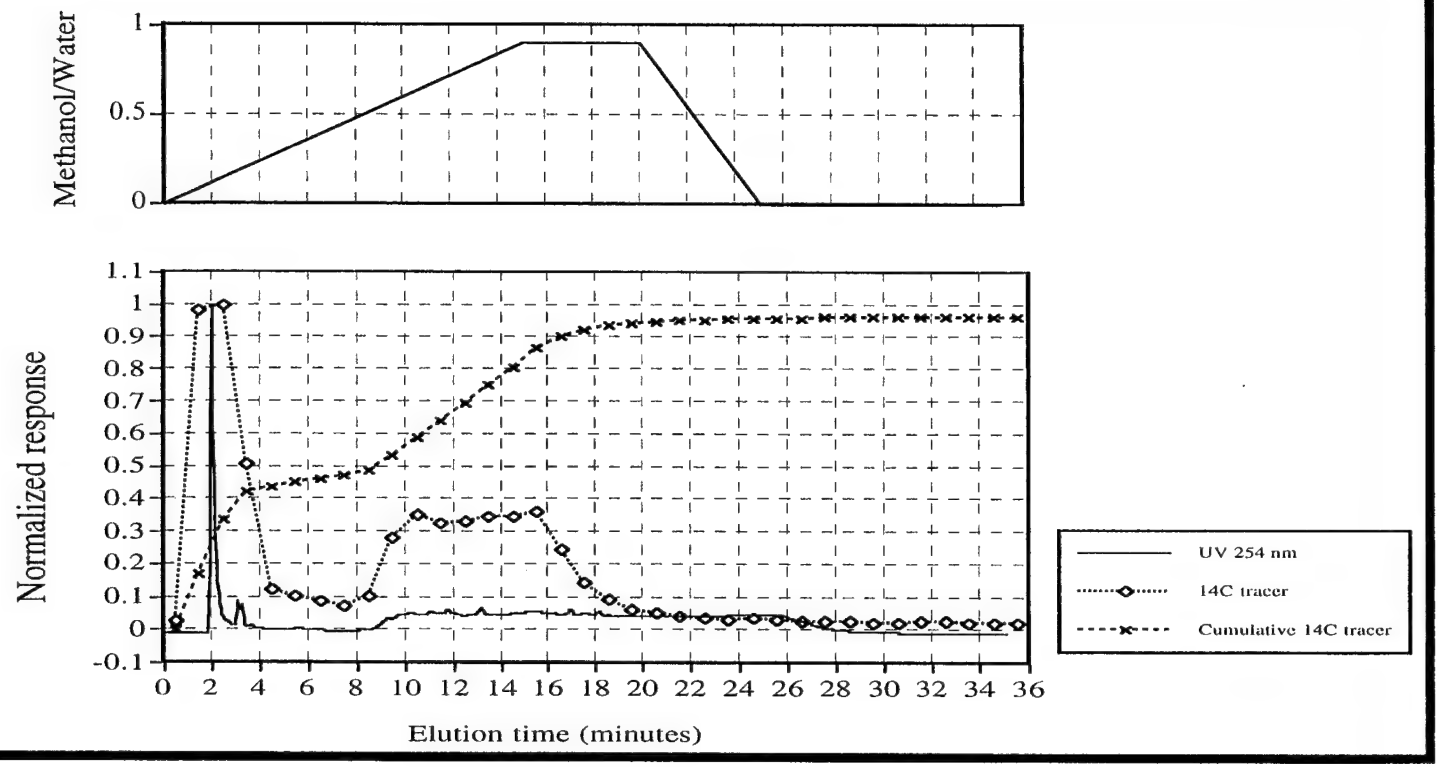
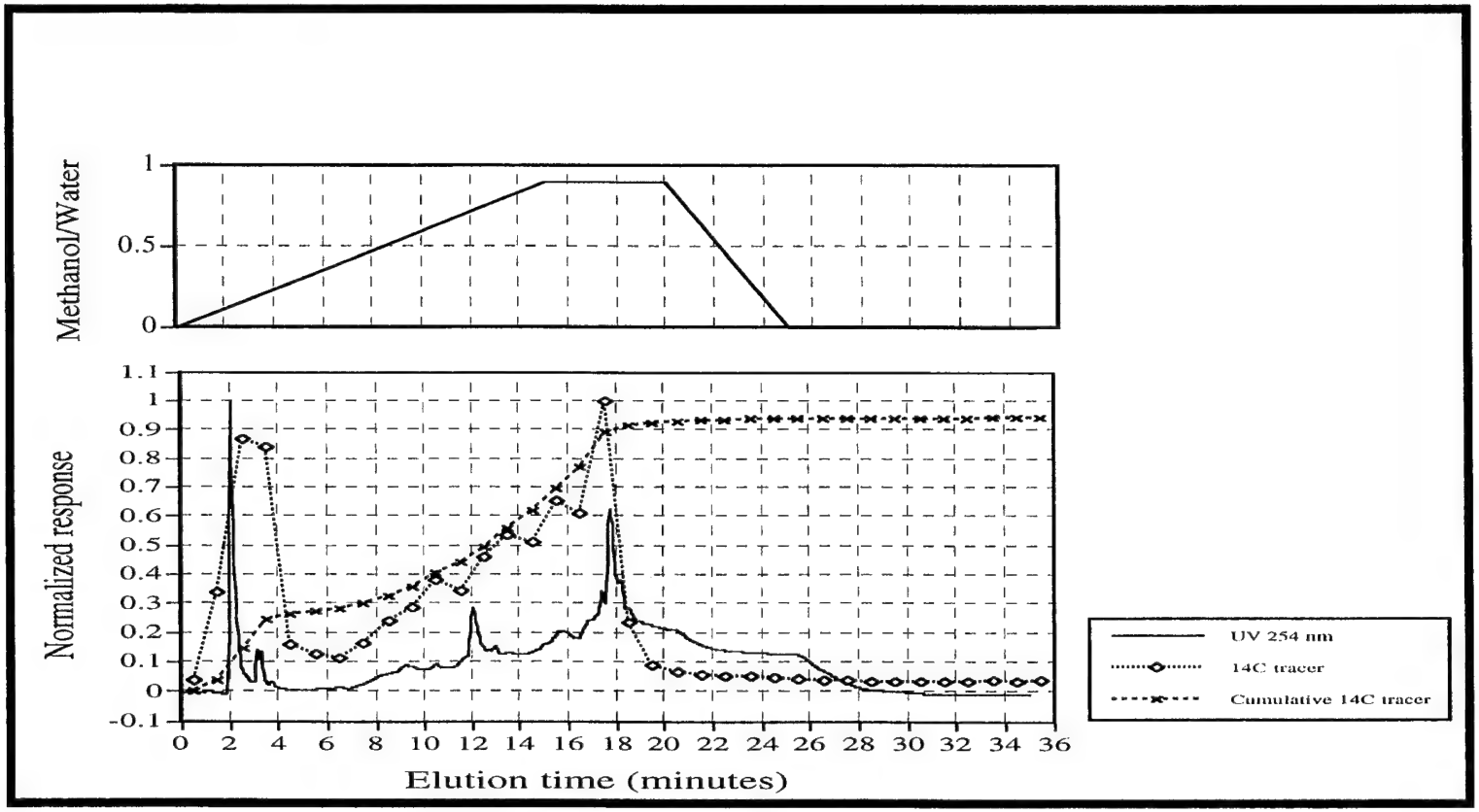
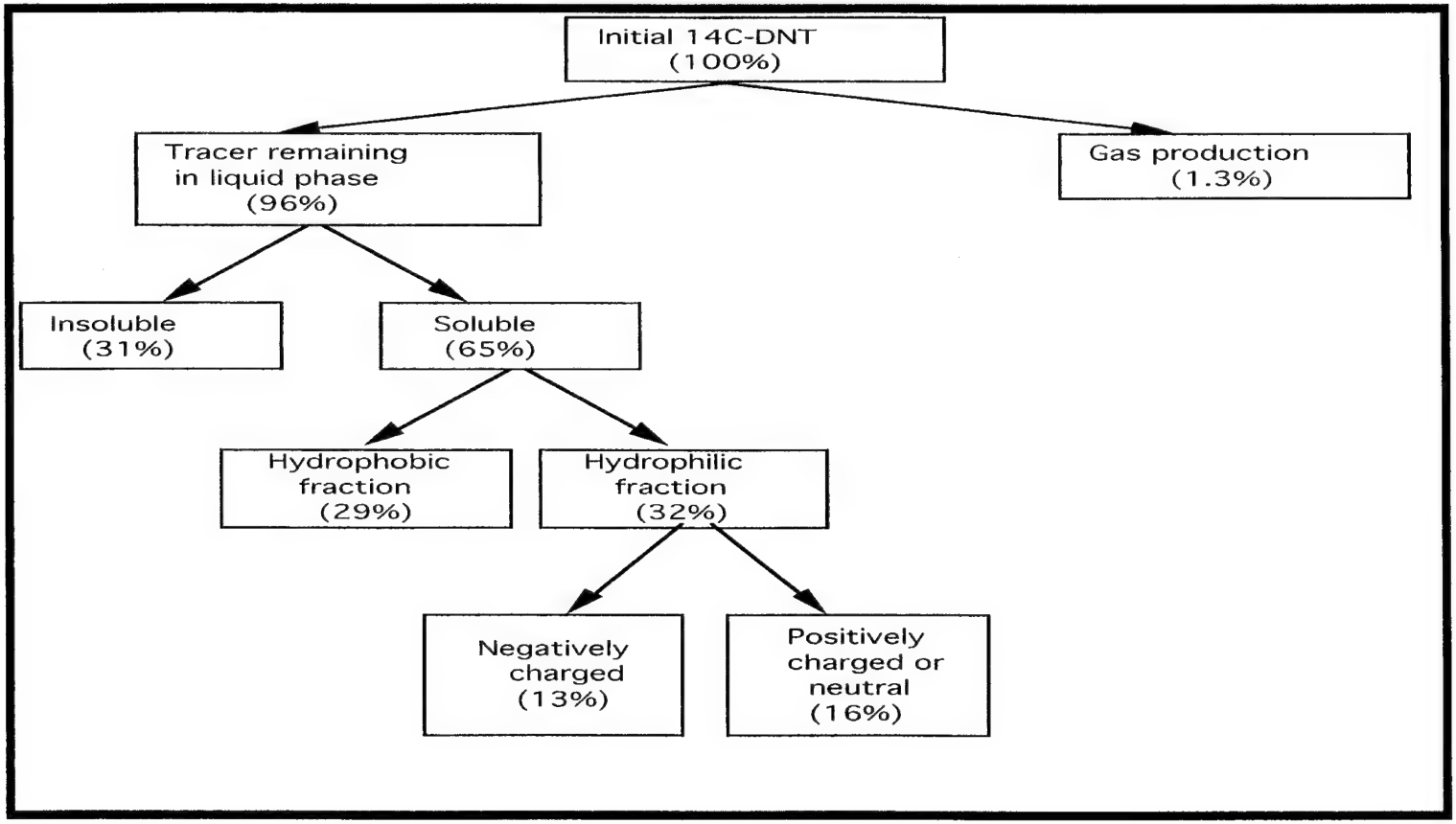


**Figure 5. HPLC analysis of the soluble DNT metabolites. Gradient elution with aqueous buffer/methanol system and cetyltrimethylammonium bromide**



# Figure 6. Flow chart of the characterization of the DNT metabolites using the 14C-DNT tracer



## 2.2 Characterization of the Denitrifying Microbial Community

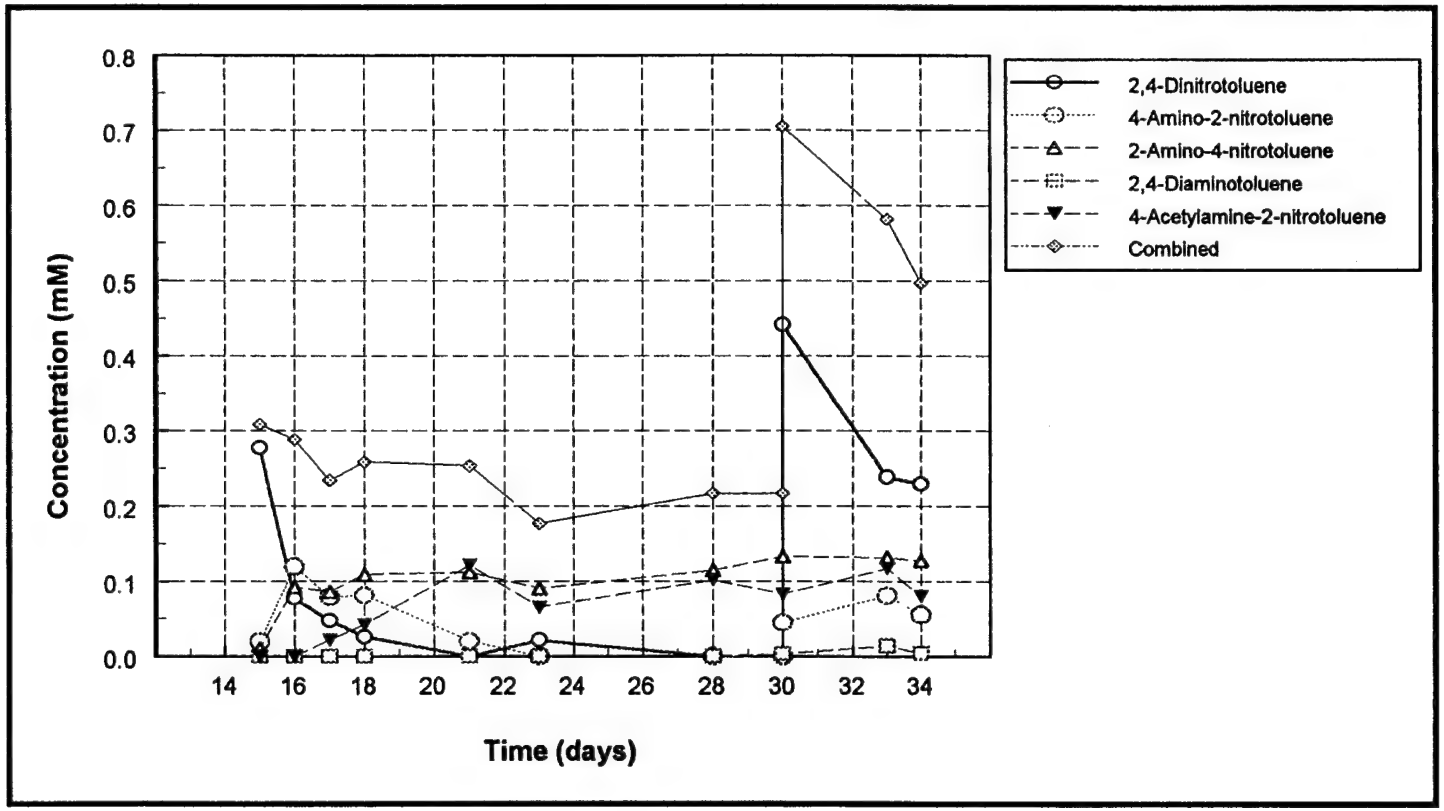
In order to isolate and identify the microorganisms responsible for the biotransformation of DNT, samples from the denitrifying enrichment cultures were aseptically transferred to solid medium. The medium was prepared by adding 8.9 g of agar to 500 ml of the liquid medium used in the enrichment cultures. Petri dishes incubated aerobically showed growth of two morphologically distinct colonies: fast-growing colonies of Gram (-) rods and slow-growing colonies of rod-shaped bacteria for which the Gram stain was not conclusive. The fast-growing colonies were isolated and purified, and sent to a commercial laboratory (The MiL Inc., St. Louis, MO) for identification. Using the GC-FAME and Biolog™ tests, the microorganism was identified as *Pseudomonas aeruginosa*. Some Petri dishes were incubated anaerobically using a GasPak™ disposable anaerobic system with GasPak™ envelopes used to generate an atmosphere of $CO_2$ and $H_2$. The growth of colonies was similar to what was observed on aerobically incubated dishes. The fast-growing microorganisms were again isolated and identified (The MiL Inc., St. Louis, MO) as *P. aeruginosa*. Isolation and identification of the slow-growing colonies is still in progress.

## 2.3 DNT Biotransformation by *P. aeruginosa*

The culture of *P. aeruginosa* was evaluated for its ability to biotransform DNT under both aerobic and denitrifying conditions. The most significant result is the inability of *P. aeruginosa* to completely reduce DNT to DAT. This is in contrast to the mixed culture from which the fast growing *P. aeruginosa* was isolated.

Figure 7 shows the results for biotransformation of DNT by *P. aeruginosa* under denitrifying conditions. As with the denitrifying enrichment cultures, the initial attack on DNT involved the reduction of one nitro group and formation of aminonitrotoluene isomers. However, the subsequent reduction of the second nitro group to form DAT was not observed. Rather, *P. aeruginosa* mediates the acetylation of 4-amino-2-nitrotoluene, resulting in 4-acetylamine-2-nitrotoluene. *P. aeruginosa* apparently lacks the ability to further reduce 2-amino-4-nitrotoluene. After 30 days of incubation, 2-amino-4-nitrotoluene plus

# Figure 7. Biotransformation of DNT under denitrifying conditions by *Pseudomonas aeruginosa*



4-acetylamine-2-nitrotoluene accounted for 45% and 35% of the original DNT added, respectively. The fate of the remaining 20% of the original DNT is still under investigation.

Figure 8 presents the biotransformation of DNT by *P. aeruginosa* under aerobic conditions. The transformation pathway is essentially the same as observed under denitrifying conditions. After 49 days of incubation, 2-amino-4-nitrotoluene and 4-acetylamino-2-nitrotoluene accounted for 25% and 32% of the added DNT, respectively. Small amounts of DNT (3%) and 4-amino-2-nitrotoluene (5%) were also detected on day 49.

[$^{14}$C]DNT was used to further characterize the fate of DNT under aerobic conditions. The tracer was added on day 30, along with a new addition of unlabeled DNT (Figure 8). Figure 9 summarizes the DNT biotransformation products formed after 18 days of incubation with the tracer. After 28 days of incubation with the tracer present, 96% of the $^{14}$C remained in the liquid culture, indicating that mineralization was minimal or nonexistent. When a sample of the culture was mixed with methanol and filtered (0.45 μm), 90% of the initial $^{14}$C added was present in the filtrate.

This soluble fraction was further characterized by a combination of HPLC and $^{14}$C tracer analyses. Fractions of the eluent from a gradient elution run were collected every 30 seconds and analyzed by scintillation counting. As described above, metabolites poorly retained in the reverse phase column are relatively hydrophilic, while metabolites with a large retention time are hydrophobic. The eluent fractions were grouped into five categories, depending on the retention times of known metabolites. A description of each group is given below and is summarized in Figure 9:

Group 1: This represents the poorly retained or unretained metabolites, and accounts for 7.3% of the original radioactivity. These are very hydrophilic materials that do not interact with the highly hydrophobic reverse phase column. In addition, due to their hydrophilicity, they are poorly extracted in methylene chloride.

# Figure 8. Biotransformation of DNT under aerobic conditions by *Pseudomonas aeruginosa*

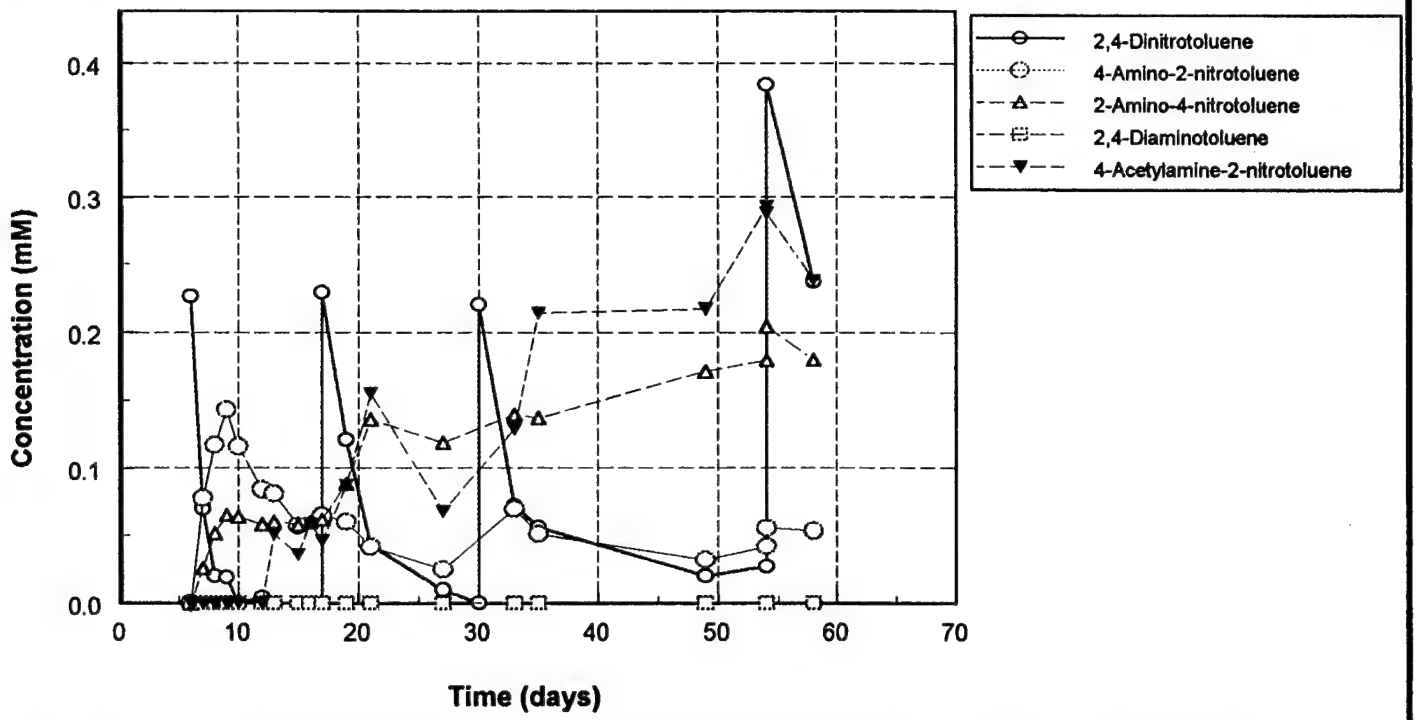
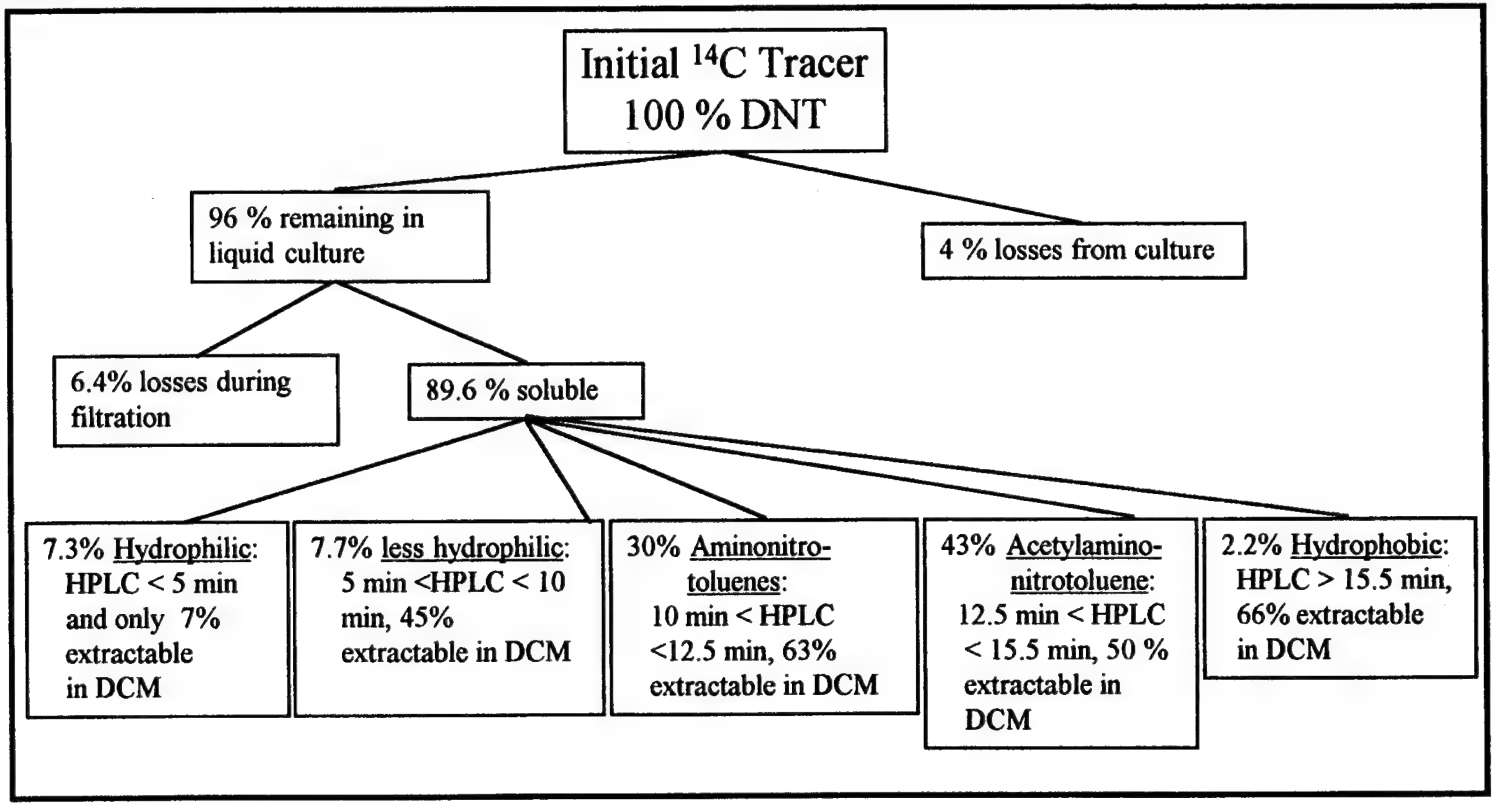


Figure 9. Characterization of DNT metabolites using the $^{14}C$-DNT tracer. Aerobic culture of *Pseudomonas aeruginosa*



Group 2: This corresponds to less hydrophilic compounds, which have a retention time between 5 and 10 minutes. They constitute 7.7% of the original tracer, with an efficiency of extraction in methylene chloride of 45%.

Group 3: The third group corresponds to the retention time of the aminonitrotoluene isomers (11 min), and accounts for 30% of the original tracer. Most of this radioactivity is associated with the accumulation of 2-amino-4-nitrotoluene.

Group 4: This includes DNT and 4-acetylamine-2-nitrotoluene, which coelute at a retention time of 13.2 min. The amount of tracer associated with this fraction (43%) should mainly correspond to the accumulation of 4-acetylamine-2-nitrotoluene, since GC results indicated very little remaining DNT.

Group 5: The last group corresponds to very hydrophobic metabolites, with retention times greater than 15 min. Only 2.2 % of the original tracer was associated with this fraction.

This characterization of DNT transformation under aerobic conditions indicates that *P. aeruginosa* is unable to completely reduce DNT. Most of the tracer ended up as 2-amino-4-nitrotoluene and 4-acetylamine-4-nitrotoluene, without further transformation. Although a $^{14}C$ tracer study has not yet been done with *P. aeruginosa* under denitrifying conditions, the GC results also demonstrated the accumulation of 2-amino-4-nitrotoluene and 4-acetylamine-2-nitrotoluene. Therefore, the complete reduction of DNT to DAT observed in the denitrifying enrichment culture was most likely due to the combined effort of the different microorganisms present. As described above, work is in progress to isolate and characterize the slow-growing organisms found in the denitrifying enrichment cultures.

## 2.4 Conclusions

1. DNT is readily biotransformed under denitrifying conditions.
2. 2-Amino-4-nitrotoluene, 4-amino-2-nitrotoluene, and 2,4-diaminotoluene were identified as intermediate metabolites during the biotransformation of DNT by the denitrifying enrichment cultures. However, the maximum amount of DAT recovered represented only 80% of the DNT added.

3. After 80 days of incubating the enrichment culture with DNT, none of the original DNT remained as aminonitrotoluenes or DAT. The formation of other metabolites was not observed during GC analysis, and with HPLC analysis, the unidentified metabolites were not retained in the reversed phase column.

4. Analysis of the liquid and gas phases of the enrichment cultures indicated that 96% of the $^{14}C$ added remained in the liquid phase after 180 days of incubation. Approximately 35% of the tracer ended up as insoluble material. Only 12-18% of the soluble material was extractable in methylene chloride or ether. From HPLC analysis, 45% of the soluble metabolites were hydrophilic and 50% were hydrophobic. The hydrophobic material seemed to be a combination of several metabolites at very low concentrations. The hydrophilic fraction seemed to be a combination of negatively charged metabolites (25% of the soluble metabolites) plus neutral or basic metabolites (20% of the soluble metabolites).

5. The denitrifying enrichment culture contained two morphologically different colonies: A fast-growing Gram (-) rod and a slow-growing rod with inconclusive Gram stain results. The fast-growing microorganisms were identified as *P. aeruginosa*. Work is in progress to isolate and identify the slow growing organisms.

6. *P. aeruginosa* can only partially reduce DNT to DAT under both aerobic and denitrifying conditions. Accumulations of 2-amino-4-nitrotoluene accounted for 25% and 45% of the DNT added under aerobic and denitrifying conditions, respectively, and the accumulation of 4-acetylamino-2-nitrotoluene accounted for 32% and 35% under aerobic and denitrifying conditions, respectively.

## 3.0 LIST OF PUBLICATIONS AND TECHNICAL REPORTS

The results from this project will be presented in the following ways:

- Mr. Noguera will be giving a poster session at the 95th General Meeting of the American Society for Microbiology, May 21-25, 1995, Washington, D.C. The title of the poster is

"Characterization of the Metabolites Produced During the Biotransformation of 2,4-Dinitrotoluene." The abstract will be published in the Proceedings of the Meeting.

- Mr. Noguera will be giving a platform presentation at the 68th Annual Water Environment Federation Conference, October 21-25, 1995, Miami Beach. A paper will be published in the Conference Proceeding, entitiled "Aerobic and Anoxic Biotransformation of 2,4-Dinitrotoluene by *Pseudomonas aeruginosa* ."
- The results from this research will be included in Mr. Noguera's doctoral dissertation.

## 4.0 LIST OF PARTICIPATING SCIENTIFIC PERSONNEL

- Dr. David L. Freedman served as the principal investigator for this project.
- Mr. Daniel R. Noguera served as the graduate research assistant, and he was responsible for conducting the research.

## 5.0 REPORT OF INVENTIONS

None to report.

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## 7.0 APPENDIX 1: Methods

### 7.1 Analytical Methods

Nitrite and nitrate were measured by ion chromatography. During the early stages of the study, a 150 mm Alltech Universal Anion/R column was used with glucose borate/gluconate buffer as the mobile phase. The run was isocratic at 1.5 ml/min. Later, a Waters Anion column was employed. The mobile phase was borate/gluconate buffer, with an isocratic run at 1.2 ml/min. In both cases, detection was done at UV 214 nm.

The concentration of ethanol was determined by measuring the presence of ethanol in the gas phase of the cultures. A 0.5 ml headspace sample was injected into a GC equipped with a J&W DB-1 megabore chromatography column and a FID for detection of metabolites. Because of the small Henry's law constant for ethanol, the response factor on the GC was independent of the culture's gas and liquid volumes. As a result, ethanol concentrations could also be measured by withdrawing liquid samples (5 ml), placing these in small serum vials (17 ml), and sampling their headspace once equilibrium was reached.

The concentration of aromatic compounds was measured by either HPLC or GC. A reversed phase HPLC column (Bio-Rad Hi-Pore RP-318) was used for the detection of DNT, aminonitrotoluenes and DAT. These compounds were isocratically separated using a 50/50 methanol/water + 1% triethylamine mobile phase at 1.5 ml/min at 35 °C (2-Amino-4-nitrotoluene and 4-amino-2-nitrotoluene were not separated under these conditions). Detection was performed with UV at 254 nm. Gradient elutions with an aqueous buffer/methanol system were used to characterize the relative hydrophobicity of the unidentified metabolites. The elutions consisted of four parts: First, a linear gradient that increased the concentration of the organic modifier from 0 to 90% in 15 minutes. Second, the concentration of ethanol was maintained at 90% for 5 min. Third, a 5 min reversed gradient returned the eluent to 100% aqueous, and finally, a 10 min equilibration period with 100% aqueous eluent. The buffer was made with 15 mM triethylamine titrated to pH 2.5 with phosphoric acid.

Gas chromatography was also used for the detection of aromatic compounds. Separation of the aminonitrotoluene isomers was easily achieved. Either a J&W DB-624 or a J&W DB-1 Megabore column (30m x 0.53 mm I.D.; Film 3mm) was used for this purpose. Helium was used as the carrier gas (30 cm/sec), and FID was used for detection. With the DB-624 column, the temperature program was: 145°C for 5 minutes, then 30°C/min to 200°C, hold for 15 minutes. The temperature program used with the DB-1 column was: 80 °C for 5 min, 30°C/min to 180°C, hold for 5 min, 30°C/min to 230°C, hold for 15 minutes.

## 7.2 Sample Preparation

Samples from the cultures (2 ml each) were mixed with 2 ml of methanol prior to filtration (0.45 μm PTFE) and analysis of aromatics by HPLC. An additional extraction in methylene chloride or chloroform was made for samples analyzed by GC and GC-MS. Samples for nitrate and nitrite analysis on the HPLC were prepared by diluting the filtered methanol/culture mixture 50 fold, or by diluting the original culture 100 fold.